

Original article

Functional characterization of the *mraW* RNA methyltransferase in growth and cell survival of *Mycobacterium smegmatis*

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Abstract

Background: Tuberculosis remains a major global health threat, exacerbated by the increasing prevalence of drug-resistant strains. The RNA methyltransferase *mraW* is predicted to play an important regulator of bacterial gene expression and cellular physiology. However, its function in mycobacteria remains unclear.

Objective: This study aims to characterize the function of the *mraW* RNA methyltransferase in *Mycobacterium smegmatis* using a CRISPR interference (CRISPRi)-mediated gene knockdown.

Methods: A *mraW*-targeting sgRNA was constructed and expressed in *M. smegmatis* mc²155, and knockdown effect was induced using anhydrotetracycline (ATc). Knockdown efficiency was confirmed by RT-qPCR, which showed approximately 55% reduction in *mraW* transcript levels.

Results: Phenotypic analysis revealed that *mraW* knockdown resulted in ATc concentration-dependent growth inhibition, reduced colony formation, and decreased bacterial viability in both solid and liquid culture conditions. Consistent with the inducible nature of the CRISPRi system, stronger growth defects were observed at higher ATc concentrations, suggesting increased repression of *mraW*, while pLJR962 vector controls remained unaffected, confirming that the observed effects were specifically associated with *mraW* knockdown. These findings demonstrate that *mraW* is important for optimal growth and survival in *M. smegmatis*, supporting a role for RNA methyltransferases in essential mycobacterial physiological processes.

Conclusion: This study further highlights the utility of CRISPRi for functional characterization of essential genes in mycobacteria and suggests that RNA methylation pathways may represent novel potential targets for future anti-tuberculosis strategies.

Keywords: CRISPRi, *Mycobacterium smegmatis*, *mraW*, RNA methyltransferase.

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Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* and remains one of the leading causes of illness and death worldwide. Despite global preventive strategies over the past decade, which reduced TB-related deaths by half, the disease remains a global concern.^(1, 2) A major challenge in controlling tuberculosis is the increasing prevalence of drug resistance, including multidrug-resistant TB (MDR/RR-TB) and extensively drug-resistant TB (XDR-TB). WHO Global Tuberculosis report 2024 has reported the treatment success rate for drug-resistant TB only 60%–68% during 2019–2021. In 2023, approximately new 159,684 cases of MDR-TB and 28,982 cases XDR-TB have been reported worldwide.⁽¹⁾ Treatment of drug-resistant TB requires prolonged therapeutic regimens and is often associated with substantial toxicity and unfavorable clinical outcomes.^(1,3) Therefore, the limited treatments efficacy and increasing drug-resistant tuberculosis highlight the urgent need for improved strategies for its management and control.

Epitranscriptomics, the study of chemical modifications on RNA has emerged as a rapidly expanding field in recent years, highlighting RNA modifications as a critical regulatory layer of gene expression. These RNA modification's function serves as key post-transcriptional modulators that influence RNA stability, translation efficiency, and cellular stress response.^(4, 5) Although extensively studied in eukaryotes, RNA modifications are also widespread in bacteria^(4, 6), where they contribute to essential cellular processes and support bacterial survival under diverse environmental conditions.^(4, 6-8)

In bacteria such as *Escherichia coli*, RNA modifications have been well characterized.⁽⁹⁻¹¹⁾ Within rRNA modification, the 16S rRNA of *E. coli* contains several modifications clustered in the P-site decoding center, which are crucial for maintaining local rRNA structure, RNA–RNA interactions, 30S subunit assembly, and antibiotic resistance.^(12, 13) The methyltransferases *rsmH* (*mraW*) and *rsmI* (*yraL*) were identified through reverse genetics combined with LC/MS-based ribonucleome analysis⁽¹²⁾ and were responsible for the N4 and 2'-O-methylation (m⁴Cm) of C1402 in the 16S rRNA.^(12, 13) Loss of this m⁴Cm modification by knockout of *rsmH* (*mraW*) and *rsmI* (*yraL*) methyltransferases significantly affected *E. coli* growth across different temperature conditions⁽¹²⁾ and also led to motility defect linked to methylation of promoter regions regulating

flagellar gene expression.⁽¹³⁾ However, this motility-associated mechanism is unlikely to be conserved in mycobacteria do not process flagella.

Functionally, *mraW* is the second open reading frame (ORF) in the division and cell wall (*dcw*) cluster and encodes a cytoplasmic methyltransferase associated with cell division processes.⁽¹⁴⁾ Although *mraW* had been studied in *E. coli*, where it effects on growth, and motility.⁽¹²⁾ However, the roles of *mraW* in RNA methylation and cell survival remains unclear in mycobacteria. This gene represents a particular interesting target because it is associated with both RNA modification and the division and cell wall (*dcw*) gene cluster, which is important for mycobacterial survival. *M. smegmatis*, a non-pathogenic model organism for *M. tuberculosis*, provides a suitable system to investigate *mraW* function and its potential as a therapeutic target. Targeting bacterial-specific RNA methyltransferases may offer a promising strategy for developing selective anti-tuberculosis drugs, particularly for drug-resistant TB where treatment efficacy is limited and regimens remain prolonged and toxic.

Therefore, this study aims to characterize the roles of the *mraW* RNA methyltransferase in *M. smegmatis* by CRISPR interference (CRISPRi)-mediated gene knockdown to assess its impact on mycobacterial growth and RNA methylation. This research determined *mraW* contributes to growth and characterize the role in RNA methylation. These findings were provided a comprehensive understanding for the roles and essentiality of *mraW* RNA methyltransferase in *M. smegmatis*.

Materials and methods

Mycobacterial growth and culture conditions

M. smegmatis strain mc²-155 (ATCC 700084) was obtained from Department of Microbiology, Faculty of Medicine, Chulalongkorn University. Mycobacterial cells were cultured on Middlebrook 7H10 agar (7H10 agar) or in Middlebrook 7H9 broth (7H9 medium) (Himedia, Maharashtra, India) supplemented with 0.25% (v/v) glycerol and 10% ADC (0.05% oleic acid, 5% BSA, 2% dextrose, 0.0004% catalase, and 0.85% NaCl). For liquid cultures, 0.25% (v/v) Tween 80 was added. Cultures were incubated at 37 °C with shaking at 200 rpm. For cloning experiments, *E. coli* DH5α was grown in Luria Bertani (LB) broth or agar at 37 °C. Kanamycin (cat#GA9089, Glentham, UK) was added at

concentrations of 50 µg/mL for *E. coli* and 25 µg/mL for *M. smegmatis* mc²-155. Anhydrotetracycline (ATc, cat#37919, Sigma-Aldrich, St. Louis, MO, USA) was added when required to induce CRISPRi-mediated gene knockdown.⁽¹⁵⁻¹⁷⁾

Identification of essential RNA methyltransferase in mycobacteria

The *mraW* gene of RNA methyltransferase was identified using bioinformatics approaches comparable database tools. Protein sequences from *M. tuberculosis* were first screened in the UniProt database to identify genes predicted to encode methyltransferase. Computational domain prediction tools were applied to compile a list of candidate genes. Transposon sequencing (Tn-seq) datasets were then examined to determine gene essentiality and to identify *mraW* as a potential essential gene associated with mycobacterial growth.

Cloning of sgRNA oligos for CRISPRi knockdown of *mraW*

CRISPRi-mediated knockdown of *mraW* (MSMEG_4235) in *M. smegmatis* was performed using the CRISPRi vector pLJR962 (Addgene no. 115162) that expresses the Sth1 dCas9 and targeting sgRNA. The anhydrotetracycline (ATc) induces the CRISPRi system, leading to the suppression of the target gene and the expected phenotypic growth characteristics. The sgRNA was designed according to dCas9Sth1 protospacer adjacent motif (PAM) sequence was selected; PAM 5'-NNGAAGG-3' based on the reference mycobacterial gene sequence.⁽¹⁸⁾ The following *mraW* CRISPRi knockdown small guide RNA (sgRNA) oligonucleotides (20-24 bp) were used:

- *mraW*_T: 5'-GGGAGGTCGAGTCCGATGAGGCGCAGCGC-3'
- *mraW*_B: 5'-AAAGGCGCTGCGCCTCATCGGACTCGAC-3'

Off-target effects were evaluated using BLAST against the *M. smegmatis* mc²-155 genome. The process began with the annealing and phosphorylation of the top and bottom sgRNA oligonucleotides to create a double-stranded fragment and subsequently ligated into the pLJR962 vector. The constructed plasmid was transformed into *E. coli* DH5α and selected LB agar with 50 µg/mL kanamycin. Transformed cells were verified by colony PCR, and plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, USA). Finally, the plasmid construct was confirmed by sanger sequencing.^(15, 17)

Electroporation of CRISPRi constructs and validation of *mraW* essentiality

A single colony of *M. smegmatis* mc²-155 was cultured in 7H9 medium with 0.05% tween 80 and ADC at 37 °C for two days. The stationary-phase culture was subsequently subcultured and incubated at 37 °C for approximately 18 h until the OD₆₀₀ reached 0.8–1.0. Cells were then harvested and washed thoroughly with 10% glycerol to prepare electrocompetent cells. The extracted plasmid was electroporated into *M. smegmatis* mc²-155 cell using Gene Pulser Xcell modular electroporation system (Bio-Rad Laboratories, England), under the following conditions : 2500 V, 700 W, 25 µF.

Following electroporation, the transformed cells were recovered in 7H9 medium with 0.05% tween 80, ADC and 25 µg/mL kanamycin at 37°C for 3–4 hours and then plated onto 7H10 agar with glycerol, ADC, and 25 µg/mL kanamycin. Plates were incubated at 37°C for 3 days or until visible colonies.⁽¹⁵⁻¹⁷⁾

To evaluate *mraW* knockdown, the *M. smegmatis* mc²-155 transformant was cultured to mid-log phase growth. The mid-log phase culture were diluted to OD₆₀₀ of 0.1 and 5 µL of 10-fold serial dilution (10⁻¹–10⁻⁵) were spotted onto 7H10 agar with glycerol, ADC, 25 µg/mL kanamycin and varying concentration of anhydrotetracycline (ATc) ; 0, 0.25, 0.5, 1 ng/mL. Then, the cultures were incubated at 37 °C for 3 days or until visible colonies. That confirmed the knockdown of the target gene, which was expected to result in a specific phenotype associated with reduced growth on agar plates. The ATc induces the CRISPRi system, leading to the suppression of the target gene and the expected phenotypic growth characteristics. All experiments were conducted in biological and technical triplicates.^(16,17)

Growth curve and cell viability of *mraW* knockdown strain

To evaluate the impact of *mraW* RNA methyltransferase knockdown to *M. smegmatis* growth. For growth curve, the *mraW*_KD and pLJR962 control was cultured in 7H9 medium with 0.05% tween 80, ADC, and 25 µg/mL kanamycin, then the culture was incubated at 37 °C with shaking 200 rpm and grown to mid-log phase (OD₆₀₀ ≈ 0.5–0.8). The mid-log phase culture was diluted to OD₆₀₀ of 0.1 and induced with various concentrations of ATc. Bacterial growth was monitored by measuring optical density at 600 nm (OD₆₀₀) at regular intervals over 0–96 h. As for growth viability assessment, the mid-log phase

culture was diluted to OD₆₀₀ of 0.1 and 20 μ L of 10-fold serial dilution (10^{-1} – 10^{-5}) was plated on 7H10 agar with glycerol, ADC, 25 μ g/mL kanamycin and varying concentration of ATc which provided optimal growth of *M. smegmatis*. To further quantify the effect of *mraW* RNA methyltransferase knockdown, colony forming unit (CFU) counts were performed at different time points from 0–96 h. All experiments were conducted in biological and technical triplicates.^(16,17)

Quantification of *mraW* gene expression by RT-qPCR

Following the knockdown of gene candidate using CRISPRi, the effect on the expression of *mraW_KD* gene was evaluated using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted and used for cDNA synthesis with 200U Reverse Aid (NEB, USA), a reverse transcriptase enzyme. Furthermore, RT-qPCR was performed using the Luna qPCR Master Mix (NEB, USA) along with specific qPCR primers designed for the target genes to measure the expression level. The housekeeping gene *sigA* was used as an internal reference for normalization. Relative gene expression levels were calculated using $2^{-\Delta\Delta C_t}$ method. All experiments were conducted in biological and technical triplicates.^(16,17)

Results

Validation of *mraW* essentiality in *M. smegmatis*

To investigate the functional role of *mraW* (MSMEG_4235) in *M. smegmatis*, a CRISPRi-mediated knockdown strain (*mraW_KD*) was established using an sgRNA specifically targeting the coding region of *mraW*. BLAST analysis against the *M. smegmatis* mc²155 genome was used to evaluate the specificity of the sgRNA, which no significant homologous off-target sites were identified. Following ATc induction, the *mraW_KD* strain exhibited dose-dependent growth reduction across ATc concentrations ranging from 0.1 to 1 ng/mL, whereas the pLJR962 vector control strain displayed normal growth under identical conditions (**Figure 1A**). Complete growth inhibition was observed at 0.25, 0.5, and 1 ng/mL ATc. In contrast, no apparent growth defect was detected at 0.1 ng/mL. Notably, a small number of ATc-resistant strains emerged at higher ATc concentrations (0.5–1 ng/mL). Importantly, ATc induction alone showed no detectable toxicity, as indicated by the unaffected growth of the pLJR962 vector control strain (**Figure 1B**). Collectively, these findings demonstrate the effectiveness of the CRISPRi-dCas9 system for inducible of gene repression and further confirm the essential role of *mraW* in the growth of *M. smegmatis*.

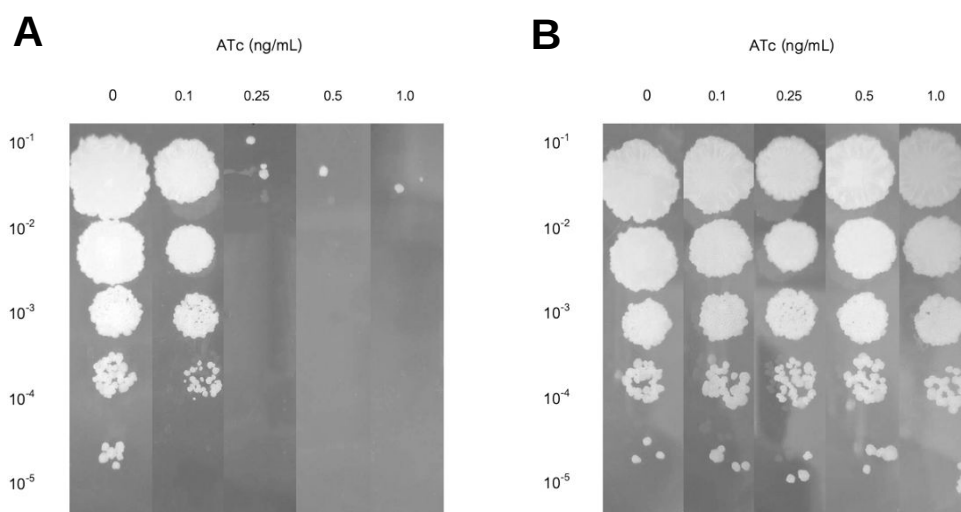


Figure 1. Effect of ATc-induced CRISPRi-mediated *mraW* knockdown on the growth of *M. smegmatis*. (A) Growth phenotype of the *mraW_KD* strain and (B) the pLJR962 vector control strain. Serial dilutions (10^{-1} to 10^{-5}) of log-phase cultures were spotted onto 7H10 agar in both the absence and presence of varying concentrations of ATc (0, 0.25, 0.5, 1 ng/mL).

Effect of ATc-induced CRISPRi-mediated *mraW* knockdown on the growth and viability of *M. smegmatis*

To evaluate the effect of *mraW* knockdown on bacterial growth and viability, the *mraW*_KD strain was induced with increasing concentrations of ATc (0–1 ng/mL). A concentration-dependent growth defect was observed following induction, as reflected by reduced optical density (OD_{600}) and bacterial viability ($\log_{10}$ CFU/mL) (**Figure 2A and B**). Compared with the uninduced control, induction with 0.25 ng/mL ATc moderately reduced bacterial growth, whereas pronounced growth inhibition was observed at 0.5 and 1 ng/mL ATc.

The growth curve of the *mraW*_KD strain showed considerable growth defect following induction with 1 ng/mL ATc, with OD_{600} remaining substantially lower than that of the uninduced culture throughout the experimental period. Significant reductions in OD_{600} were observed at 12 h ($P < 0.05$) and 24 h ($P < 0.05$). At 0.5 ng/mL ATc, bacterial growth was delayed but gradually increased over time, although these reductions did not reach statistical significance. Similarly, cultures treated with 0.25 ng/mL ATc displayed growth patterns more comparable to those of the uninduced control, particularly during prolonged incubation (**Figure 2A**).

Consistent with the growth viability was decreased following *mraW* knockdown, particularly at higher ATc concentrations. Cultures induced with 1 ng/mL ATc

exhibited reduction in bacterial viability, showing approximately a 4.9- $\log_{10}$ and 4.1- $\log_{10}$ decrease in CFU/mL at 24 h and 36 h, respectively, compared with the uninduced control, with significant reductions observed at 24 h ($P < 0.01$) and 36 h ($P < 0.05$). Although reduced viability was also observed at 0.25 and 0.5 ng/mL ATc, these effects were less pronounced. Partial recovery of cell viability was observed at later time points (72–96 h), although bacterial counts remained lower than those of the untreated control (**Figure 2B**). Collectively, these findings suggested that CRISPRi-mediated knockdown of *mraW* effect bacterial growth and viability in an ATc concentration-dependent manner, further supporting an important role for *mraW* in the growth of *M. smegmatis*.

ATc-induced CRISPRi knockdown of *mraW* expression in *M. smegmatis*

To determine whether ATc induction effectively suppressed *mraW* expression, transcript abundance in the *mraW*_KD strain was quantified by RT-qPCR. A concentration of 0.5 ng/mL ATc was selected because this concentration produced a clear growth phenotype while maintaining sufficient bacterial viability for reliable transcript analysis. In contrast, 0.25 ng/mL ATc caused only a modest growth defect, whereas 1 ng/mL ATc resulted in more severe growth inhibition.

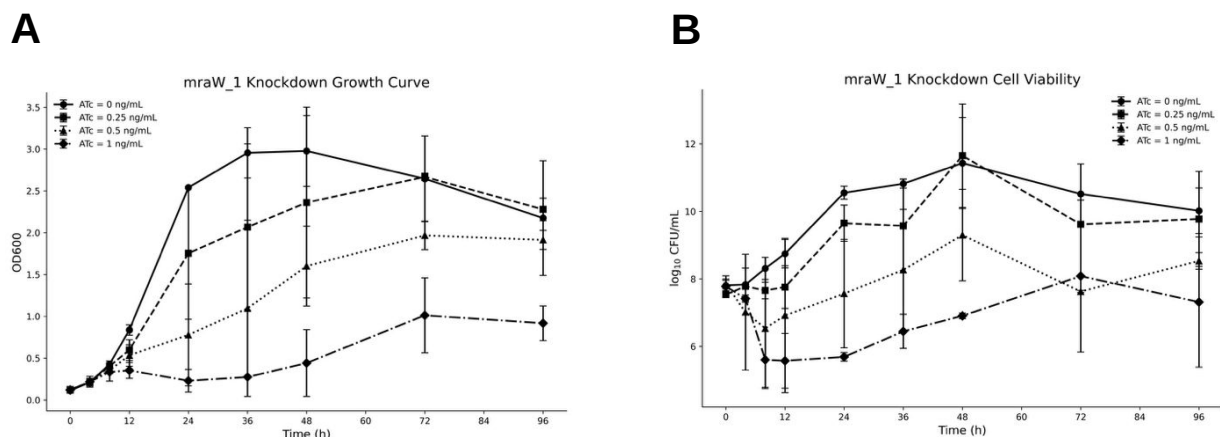


Figure 2. (A) Growth curve of the *mraW*_KD *Mycobacterium smegmatis* strain cultured in liquid medium supplemented with increasing concentrations of ATc (0, 0.25, 0.5, and 1 ng/mL), monitored by optical density at 600 nm (OD_{600}). (B) Bacterial viability (CFU) of the *mraW*_KD strain determined by $\log_{10}$ CFU/mL under the same conditions. Error bars represent standard deviation (SD).

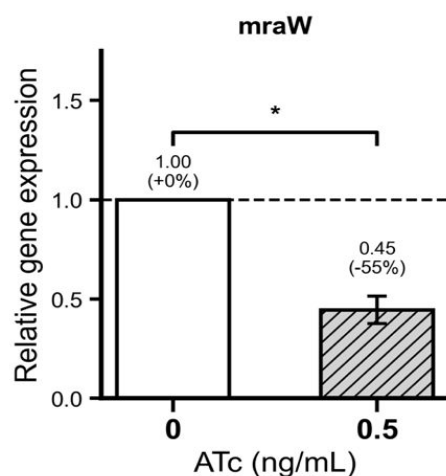


Figure 3. Relative gene expression in the *mraW*_KD strain following induction with 0 or 0.5 ng/mL ATc. Experiments were performed in biological and technical triplicates. Statistical significance is indicated as $*P < 0.05$. Error bars represent the standard deviation (SD).

Induction with 0.5 ng/mL ATc showed significantly reduced *mraW* expression, with a $55.46\% \pm 6.91\%$ reduction compared with the uninduced control ($P < 0.05$) (**Figure 3**). This *mraW* knockdown was consistent with the observed growth phenotype, supporting the effectiveness of the CRISPRi-dCas9 system for inducible gene repression in *M. smegmatis*.

Discussion

This study investigated the functional role of *mraW* (MSMEG_4235) in *M. smegmatis*, a predicted RNA methyltransferase located within the division and cell wall (*dcw*) gene cluster⁽¹⁴⁾ using a CRISPRi-mediated knockdown approach. The findings demonstrated that repression of *mraW* impaired bacterial growth in both solid and liquid culture conditions, supporting its importance in mycobacterial physiology. Growth inhibition was observed in an ATc concentration-dependent manner, with higher concentrations of ATc resulted in stronger growth suppression and reduced bacterial viability. The CRISPRi-mediated knockdown system effectively suppressed *mraW* expression, resulting in an approximately 55% reduction in transcript abundance following induction with 0.5 ng/mL ATc. This reduction was accompanied by a clear growth phenotype, including impaired colony formation, reduced OD₆₀₀, and decreased bacterial cell viability. These findings support the utility of the CRISPRi-dCas9 system as an inducible platform for functional characterization of essential genes in mycobacteria.⁽¹⁵⁻¹⁷⁾ Furthermore, the absence of detectable toxicity in the vector control strain indicates that the observed phenotype was likely attributable to *mraW* repression rather than ATc exposure alone.

The growth defect observed following *mraW* knockdown is consistent with previous observations in *E. coli*, in which deletion of *rsmH* (*mraW*) disrupted bacterial growth under different temperature conditions.⁽¹²⁾ In *E. coli*, *mraW* functions as a 16S rRNA methyltransferase responsible for N4-methylation of cytidine 1402 (m4Cm) in the decoding center of the 30S ribosomal subunit, contributing to ribosome stability, translational fidelity, and proper ribosome assembly.^(12, 13) Although the precise methylation targets of *mraW* in mycobacteria remain unclear, repression of *mraW* may impair ribosome-associated functions, thereby reducing bacterial growth and fitness. Sequence comparison further demonstrated that *mraW* of *M. smegmatis* shares 71.63% nucleotide identity (blastN) and 70.62% amino acid identity (blastP) with its homolog in *Mycobacterium tuberculosis*. This degree of conservation supports the use of *M. smegmatis* as a surrogate model for investigating *mraW* function and suggests that the physiological role of this RNA methyltransferase may be conserved among mycobacterial species. Furthermore, *mraW* is located within the conserved *dcw* gene cluster involved in cell division and peptidoglycan biosynthesis.⁽¹⁴⁾ The observed growth defect may also reflect broader physiological consequences associated with disruption of cell division related processes. Interestingly, partial recovery of growth and viability at later time points may indicate incomplete knockdown or adaptive responses under prolonged CRISPRi induction, although the underlying mechanisms remain to be further investigated.

Overall, the present findings suggest that *mraW* contributes to optimal growth and viability in *M. smegmatis*, supporting its importance in mycobacterial physiology. Although the precise molecular mechanisms underlying the observed phenotypes remain to be fully characterized, repression of *mraW* may affect RNA methylation, thereby impairing bacterial growth. Future studies examining survival-associated phenotypes following *mraW* knockdown, including effects on stress adaptation, cell morphology, and antibiotic susceptibility will further clarify the physiological importance of *mraW* in mycobacteria. Given the increasing need for novel therapeutic targets against drug-resistant tuberculosis, survival-associated genes such as *mraW*, which contribute to bacterial growth and adaptation, may represent promising targets for future therapeutic development.

Conclusion

This study demonstrates that CRISPRi-mediated knockdown of *mraW* reduced bacterial growth and viability in *M. smegmatis* in an ATc concentration-dependent manner. *mraW*_KD significantly decreased gene expression and resulted in impaired colony formation, delayed growth, and decreased bacterial viability. These findings support an important role of *mraW* in mycobacterial growth and provide preliminary evidence that RNA methyltransferases contribute to essential physiological processes in mycobacteria. Further investigation of *mraW* functions in RNA methylation and cell survival phenotypes may improve understanding of mycobacterial biology and support the identification of potential therapeutic targets.

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Conflicts of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

Statistical summaries are presented in the published article. The data sets generated or analyzed during the present study are available from the corresponding author on reasonable request.

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